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(71) Applicant: CALIFORNIA INSTITUTE OF BIOLOGICAL RESEARCH [US/US]; 11099 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventor: BOURDON, Mario, A. ; 11342 Calle Dario, San Diego, CA 92126 (US).

(74) Agent: BINGHAM & FITTING; 12526 High Bluff Drive, Suite 300, San Diego, CA 92130 (US).

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(54) Title: TENASCIN RELATED PEPTIDES

(57) Abstract

A polypeptide of the formula: X-Ser-Arg-Arg-Gly-Asp-Met-Ser-Z wherein X is a chain of from 1 to 20 amino acid residues or an amino-terminal group and Z is a chain of from 1 to 20 amino acid residues or a carboxy-terminal group. The polypeptide mimics the ability of tenascin to promote cell attachment.

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TENASCIN RELATED PEPTIDES

DESCRIPTION

Technical Field

The present invention relates to polypeptides and anti-polypeptide antibodies capable of modulating cell attachment to tenascin. More specifically, the polypeptides and antipolypeptide antibodies of this invention can be used to inhibit tumor metastasis and angiogenesis. The polypeptides are further capable of mimicking the ability of tenascin to promote cell attachment.

Background

Tenascin is a multifunctional extracellular matrix glycoprotein with an oncodevelopmental pattern of expression, Erickson, Annu. Rev. Cell Biol., 5:71-92, 1989. First described as a tumor associated matrix protein, Bourdon et al., Cancer Res., 43:2796-2805, 1983, tenascin is expressed in malignant tumors such as glimas, melanomas, and breast carcinomas, Bourdon et al., Cancer Res., 43:2796-2805, 1983; Mackie et al., Proc. Natl. Acad. Sci. USA, 84:4621-4625, 1987. In these tumors, tenascin is associated with the extracellular matrix and the tumor neovasculature indicating a function(s) for tenascin in cell-matrix interaction during tumor growth and angiogenesis.

Tenascin expression developmentally is highly regulated and associated with mesenchymal differentiation during mesenchymal-epithelial interactions of kidney, Aufderheide et al., J. Cell Biol., 105:599-608, 1987, gut, Aufderheide et al., J. Cell Biol., 107:2341-2349, 1988, and breast, Chiguet-Ehrismann et al., Cell, 47:131-139, 1986, as well as during central nervous system development, Chuong et

al., J. Cell Biol., 104:331-342, 1987; Grumet et al., Proc. Natl. Acad. Sci. USA, 82:8075-8079, 1985; Steindler et al, Dev. Biol., 131:234-260, 1989, and neural crest cell migration, Epperlein et al., Development, 103:743-756, 1988; Bronner-Fraser, J. Neuro-Sci. Res., 21:135-147, 1988); Mackie et al., Development, 102:237-250, 1988. During these events tenascin is expressed transiently indicating that there is a set of specific functions which tenascin carries out during differentiation. Tenascin is also expressed during wound healing where its expression is also transient, Mackie et al., J. Cell Biol., 107:2757-2767, 1988.

Structurally, tenascin is a large (250-280 kd) glycoprotein which forms disulfide trimers and hexamers; these last are 1.5 to 1.8 x 10^6 daltons in size, Gulcher et al., Proc. Natl. Acad. Sci. USA, 86:1588-1592, 1989; Jones et al., Proc. Natl. Acad. Sci. USA, 85:2186-2190, 1988; Spring et al., Cell, 59:325-334, 1989. Within tenascin there are multiple fibronectin type III repeats flanked by aminoterminal EGF-like repeats and carboxy-terminal fibrinogen-like globular end. These domains likely contain specific functional sites. Indeed, the number of functions attributed to tenascin indicate tenascin is a multifunctional protein. Among the functions attributed to tenascin are cell adhesion, Bourdon et al., J. Cell Biol., 108:1149-1155, 1989, heparin-proteoglycan binding, Hoffman et al., J. Cell Biol., 106:519-532, 1988, and anti-adhesive properties, Spring et al, Cell, 59:325-334, 1989.

Brief Summary of the Invention

The present invention contemplates a polypeptide of the formula:

X-Ser-Arg-Arg-Gly-Asp-Met-Ser-Z

wherein:

X is a chain of 1 to 20 amino acid residues or an amino-terminal group; and

Z is a chain of 1 to 20 amino acid residues or a carboxy-terminal group.

Preferred polypeptides include those represented by the formulae:

NH_2-Ser-Arg-Arg-Gly-Asp-Met-Ser-COOH, and

NH_2 Ser-Arg-Arg-Gly-Asp-Met-Ser-NH_2.

In another embodiment, the present invention contemplates a method for inhibiting attachment of cells to tenascin comprising contacting the cells with a peptide of this invention in an amount of said peptide effective to inhibit said attachment.

The present invention also contemplates a method for inhibiting attachment of cells to a subcellular matrix comprising contacting the subcellular matrix with an antibody that immunoreacts with a polypeptide of the formula:

NH_2-Ser-Arg-Arg-Gly-Asp-Met-Ser-COOH,

but does not immunoreact with a polypeptide of the formula:

NH_2-Gly-Arg-Asp-Gly-Ser-Pro-COOH.

An antibody, preferably a monoclonal antibody, that immunoreacts with tenascin and an epitope formed by the sequence Ser-Arg-Arg-Gly-Asp-Met-Ser, but which does not immunoreact with the sequence Gly-Arg-Gly-Asp-Ser-Pro, is also contemplated.

Pharmaceutical compositions, preferably in unit does form, of a polypeptide or antibody of this invention are also contemplated, such compositions being useful for modulating cell attachment in vivo when administered in a therapeutically effective amount.

Brief Description of the Drawings

In the drawings forming a portion of the specification:

Figure 1 illustrates the amino acid residue sequence of a portion of tenascin and a nucleotide sequence coding for it from nucleotide sequence residue 1201 through 1600. Gulcher et al., Proc. Natl. Acad. Sci. USA, 86:1588-1592, 1989. The preferred nucleotide sequence coding for the cell interacting site of tenascin, SRRGDMS, begins at nucleotide 1433 and ends at 1451.

Figure 2 Inhibition of cell attachment to tenascin with anti-peptide antibodies. Cell attachment to wells coated with 3 μg/μl tenascin, fibronectin, or laminin was assayed in the presence of anti tenascin antibody (100 μg) or anti-SRRGDMS antibody affinity purified on tenascin (50 μg).

Figure 3 Cell attachment to SRRGDMS-BSA conjugate. Cell attachment to SRRGDMA-BSA (■) and GRGDSP-BSA (▲) or control MGSRSRD-BSA (scrambled tenascin-derived peptide) (O) were assayed in wells coated with increasing amounts of peptide conjugate.

Figure 4 Peptide inhibition of Cell Attachment. Cell attachment to tenascin (TN), plasma fibronectin (FN) and laminin (LN) was assayed in the presence of synthetic peptides (1 mg/ml final concentration). The peptide SDDYSGSGSG is derived from the glycosaminoglycan attachment signal of serglycin; the peptide GRGESP is a negative control derived from the GRGDSP fibronectin cell attachment signal; and the peptide SRRGDMS is derived from human tenascin.

Figure 5 Peptide SRRGDMS cell attachment inhibition curves. Cell attachment of human U251 cells to tenascin (O) and fibronectin (O) was assayed with increasing concentrations of SRRGDMS peptide.

Figure 6 Inhibition of cell attachment to peptide-BSA conjugates with soluble peptide. Cell attachment to either SRRGDMS-BSA (1 μg/ml coating concentration) or GRGDSP-BSA (0.5 μg/ml coating concentration) was assayed in the presence or absence of 1 mg/ml SRRGDMS-NH_2, GRGDSP-NG_2, or MGSRSRD-NH_2.

Figure 7 Anti-SRRGDMS antibody binding to peptide conjugates. Anti-SRRGDMS antibody specificity was assayed by ELISA. The anti-SRRGDMS, at 1 mg/ml IgG concentration, antibody was allowed to bind peptide-BSA conjugates (0.10 μg/ml coating concentration), washed with PBS, 0.02% Tween 20. Bound antibody was detected with goat anti-rabbit-alkaline phosphatase secondary antibody and developed with the substrate PNPP.

Figure 8 Inhibition of cell attachment to SRRGDMS-BSA by anti peptide antibody. Cell attachment to wells coated with 1 μg/ml SRRGDMS-BSA or 0.05 μg/ml GRGDSP-BSA was inhibited by both anti-SRRGDMS (50 μg) and anti-tenascin antibody (50 μg) affinity purified on SRRGDMS-sepharose. Maximum cell attachment was observed in control wells containing media alone or IgG from preimmune sera.

Detailed Description of the Invention

A. Definitions

Amino Acid Residue: The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH2 refers to the free amino group present at the amino- or carboxy- terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. The amino-terminal NH_2

group and carboxy-terminal COOH group f free polypeptides are typically not set forth in a formula. A hyphen at the amino- or carboxy-terminus of a sequence indicates the presence of a further sequence of amino acid residues or a respective NH_2 or COOH terminal group. In keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
C	Cys	cysteine

It should be noted that all amino acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus.

Polypeptide: refers to a linear series of amino acid residues connected to one another by peptide bonds between the alpha-amino groups and carboxy groups of contiguous amino acid residues.

Peptide: as used herein refers to a linear series of no more than about 50 amino acid residues connected one to the other as in a polypeptide.

Protein: refers to a linear series of greater than 50 amino acid residues connected one to the other as in a peptide.

Synthetic peptide: refers to a chemically produced chain of amino acid residues linked together by peptide bonds that is free of naturally occurring proteins and fragments thereof.

Nucleotide: a monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked nucleosides is typically referred to herein as a "nucleotide sequence", and is represented herein by a formula whose left to right orientation is in the conventional direction of 5' terminus to 3' terminus.

B. Polypeptides

In one embodiment, the present invention contemplates a polypeptide characterized by the

f rmula:

X_n-Ser-Arg-Arg-Gly-Asp-Met-B-Z_m.

B is either Ser or Glu, preferably Ser. X and Z each represent amino- and carboxy-terminal groups, respectively. The presence or absence of X is indicated by its subscript, n, which is either 0 or 1 such that when n is 0, X is not present and when n is 1 X is present. Similarly, when m is 0, Z is not present and when m is 1, Z is present. X can be an amino-terminal NH_2 group. X can also be a chain of 1 to about 20 amino acid residues that is present when n is 1 and is not present when n is 0. Z can be a carboxy-terminal COOH group or a carboxy-terminal NH_2 group. Z can also be a chain of 1 to about 20 amino acid residues that is present when n is 1 and not present when n is 0.

X and/or Z can be one of the following amino acid residue sequences:

(a) Gly-Arg-Gly-Asp-Ser-Pro,

(b) Arg-Gly-Asp-Thr, and

(c) Arg-Gly-Asp-Cys.

(d) Lys-Arg-Lys-Arg-Lys-Arg-Lys-Arg-Arg-Gly-Asp-Val

(e) His-His-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Asp-Val

(f) His-His-Lys-Lys-Lys-Lys-Lys-Lys-Arg-Gly-Asp-Val

(g) His-His-Lys-Arg-Lys-Arg-Lys-Arg-Arg-Gly-Asp-Ser

(h) His-His-Lys-Arg-Lys-Arg-Lys-Gln-Arg-Gly-Asp-Val

(i) Lys-Lys-Lys-Lys-Lys-Lys-Lys-Lys-Arg-Gly-Asp-Val

(j) His-His-Leu-Arg-Lys-Arg-Lys-Gln-Arg-Gly-Asp-Val

In another embodiment, a polypeptide of this invention has the formula:

NH_2 $\{$Ser-Arg-Arg-Gly-Asp-Met-Ser$\}_p$ COOH.

The value of p is an integer such that the homoblock polymer is soluble in aqueous 0.15 M sodium chloride. Preferably the value of p is 2 to about 6.

A pr ferred polypeptide has less than about 30 amino acid r sidues and contains a biologically activ sequence, exhibiting cell attachment activity, of the sequence

Ser-Arg-Arg-Gly-Asp-Met-Ser.

More preferred are polypeptides according to formulae:

p1 NH_2-Ser-Arg-Arg-Gly-Asp-Met-Ser-COOH, and

p2 NH_2-Ser-Arg-Arg-Gly-Asp-Met-Ser-NH_2.

Each of the polypeptides of this invention are characterized as having the ability to mimic the cell attachment-promoting activity of tenascin.

In preferred embodiments, a subject polypeptide is operatively linked to a solid matrix, such as agarose, collagen, nitrocellulose, polyester, glass, synthetic resin, long chain polysaccharide and the like. Preferably, the subject polypeptides are operatively linked to a solid matrix forming a prosthetic device, percutaneous device, vascular graft, and the like.

For topical administration, the polypeptides of this invention can be conventionally formulated into a lotion, salve, gel, colloid power, and the like.

A subject polypeptide includes any analog, fragment or chemical derivative of a polypeptide whose amino acid residue sequence is shown herein so long as the polypeptide is capable of competitively inhibiting the binding of tenascin to its receptor. Therefore, a present polypeptide can be subject to various changes, substitutions, insertions, and deletions where such changes provide for certain advantages in their use.

The term "analog" refers to any polypeptide having an amino acid residue sequence substantially identical to a sequence specifically shown herein in which one or more residues have been conservatively substituted with a functionally similar r sidue.

Examples of conservativ substitutions include the substitution f one n n-polar (hydroph bic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided that such polypeptide displays the requisite binding activity.

"Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine

may be substituted f r lysine. Polypeptides of the present invention also includ any polyp ptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is shown herein, so long as the requisite binding activity is maintained.

The term "fragment" refers to any subject polypeptide having an amino acid residue sequence shorter than that of a polypeptide whose amino acid residue sequence is shown herein.

A subject polypeptide can be prepared using the solid-phase synthetic technique initially described by Merrifield, in J. Am. Chem. Soc., 85:2149-2154 (1963). Other polypeptide synthesis techniques may be found, for example, in M. Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed., (1976) as well as in other reference works known to those skilled in the art. A summary of polypeptide synthesis techniques may be found in J. Stuart and J.D. Young, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, IL, 3d Ed., Neurath, H. et al., Eds., p. 104-237, Academic Press, New York, NY (1976). Appropriate protective groups for use in such syntheses will be found in the above texts as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, NY (1973).

In general, those synthetic methods comprise the sequential addition of one or more amino acid residues or suitably protected amino acid residues to a growing polypeptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group such as lysine.

Using a solid phase synthesis as an example, the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amid linkage with the residue already attached to the solid support. The protecting group of the amino or carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence any remaining terminal and side group protecting groups (and solid support) are removed sequentially or concurrently, to provide the final polypeptide.

C-terminal amides, such as peptides according to formula p2, can normally be synthesized at a slightly higher purity than the comparable C-terminal acid, for the reasons described below. Normally, peptides with a C-terminal acid are cleaved in 92.5% HF/7.5% anisole for one hour. This involves an "SN1" type reaction where anisole is used as a scavenger. During this type of reaction, the side chain protecting groups which are removed yield free benzyl-type carbocations which can react with other regions of the peptide (i.e., Met, Trp, and Cyc residues) instead of the anisole scavenger. These reactions can be avoided if an "SN2" type reaction is used for cleavage. This is done using a modification of Tam's "low/high cleavage" procedure (Int. J. Pept. Prot. Res., 21:57-65, 1983). Using benzhydrylamine resins (which yield C-terminal amides), this reaction cleaves the side

chain protecting groups via a milder "SN2" type reaction which involves the unimolecular transfer of the protecting groups from the peptide to the scavenger (bypassing the reactive carbocation intermediate) while leaving the peptide linked to the resin. (This method of cleavage can not be used for the much more labile resins used in the production of C-terminal acid.) The scavenger byproducts are then rinsed away and the peptide is cleaved from the resin using the standard "SN1" procedure. This usually results in at least a five percent increase in purity for most peptides and is sometimes the difference between success and failure for longer peptides or peptides containing "difficult" residues.

The polypeptides of the present invention generally contain a tenascin receptor-binding segment of at least 7 amino acid residues and up to fifty amino acid residues, preferably 10-35 amino acid residues. The polypeptides can be linked to an additional sequence of amino acids at either or both the N-terminus and C-terminus, wherein the additional sequences are from 1-100 amino acids in length. Such additional amino acid sequences, or linker sequences, are heterologous to the tenascin amino acid residue sequence and can be conveniently affixed to a detectable label, solid matrix, or carrier. Labels, solid matrices and carriers that can be used with peptides of the present invention are described below. Typical amino acid residues used for linking are tyrosine, cysteine, lysine, glutamic acid and aspartic acid, and the like. Preferred linking residues are carboxy-terminal Cys and Lys, and amino-terminal Tyr. A heparin binding sequence, such as that described by Ponez et al., Blood, 69:219-223 (1989), can also be linked to the polypeptide at either its amino or

carboxy terminus.

Any polypeptide of the present invention, including a chimeric polypeptide as described hereinbelow, may be used in the form of a pharmaceutically acceptable salt. Suitable acids which are capable of forming salts with the polypeptides of the present invention include inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, phosphoric acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, fumaric acid, anthranilic acid, cinnamic acid, naphthalene sulfonic acid, sulfanilic acid or the like.

Suitable bases capable of forming salts with the peptides of the present invention include inorganic bases such as sodium hydroxide, ammonium hydroxide, potassium hydroxide and the like; and organic bases such as mono-, di- and tri-alkyl and aryl amines (e.g. triethylamine, diisopropyl amine, methyl amine, dimethyl amine and the like) and optionally substituted ethanolamines (e.g. ethanolamine, diethanolamine and the like).

The present invention further includes a composition that includes a subject polypeptide in combination with one or more of a pH buffering agent, wetting agent, anti-oxidant, reducing agent, aqueous medium, and the like, such composition being formulated as an aqueous solution for a use as described herein or as a dry composition, such as a powder, that can be reconstituted to form an aqueous solution.

C. Chimeric Polypeptides

A chimeric polypeptide of this invention is defined by the presence of at least one tenascin

receptor-binding peptide segment defined by the formula

Ser-Arg-Arg-Gly-Asp-Met-Ser

operatively linked via a peptide bond to a peptide segment heterologous to tenascin.

The tenascin receptor-binding segments of a subject chimeric polypeptide can be either contiguous or adjacent to each other within the polypeptide chain. Where they are adjacent, the segments are separated by amino acid residues forming a spacer segment typically comprised of from about 5 conveniently up to about 50 residues, preferably about 15 to about 30 residues. A subject chimeric polypeptide can contain a plurality of the same tenascin receptor-binding segment. Where three or more of the tenascin receptor-binding segments are adjacent within a subject chimeric polypeptide, the spacer segments can be the same or different.

A subject chimeric polypeptide can further contain a head and/or tail segment of 1 conveniently up to about 50, such as about 5 or 10, typically about 15 or about 30, at its amino- or carboxy terminus, respectively, where such a segment is advantageous in the polypeptide's making or use. For instance, a tail segment can provide a means for linking the subject chimeric polypeptide to a solid matrix, where as a leader segment can advantageously be used to facilitate secretion of the polypeptide during its expression in cells.

D. DNA and Recombinant DNA Molecules

In living organisms, the amino acid residue sequence of a protein or polypeptide is directly related via the genetic code to the deoxyribonucleic acid (DNA) sequence of the gene that codes for the protein. Thus, a gene can be defined in terms of the amino acid residue sequence, i.e., protein

or polypeptide, for which it codes.

An important and well known feature of the genetic code is its redundancy. That is, for most of the amino acids used to make proteins, more than one coding nucleotide triplet (codon) can code for or designate a particular amino acid residue. Therefore, a number of different nucleotide sequences may code for a particular amino acid residue sequence. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid residue sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylations do not affect the coding relationship in any way.

The present invention contemplates a deoxyribonucleic acid (DNA) molecule or segment that defines a gene coding for, i.e., capable of expressing, a subject polypeptide or a subject chimeric polypeptide. A preferred DNA segment has a nucleotide base sequence corresponding to the sequence shown in Figure 1 from base position 1433 to 1451, 1427 to 1451, 1401 to 1451, 1427 to 1466 and 1401 to 1500.

DNA molecules that encode the subject polypeptides can easily be synthesized by chemical techniques, for example, the phosophotriester method of Matteucci et al., J. Am. Chem. Soc., 103:3185 (1981). Of course, by chemically synthesizing the coding sequence, any desired modifications can be made simply by substituting the appropriate bases for those encoding the native amino acid residue sequence. However, DNA molecules including base sequences identical to all or a portion of that shown in Figure 1 is preferred.

A DNA molecule that includes a DNA sequence encoding a subject polypeptide can be prepared by operatively linking (ligating) appropriate restriction fragments from each of the above deposited plasmids using well known methods. The DNA molecules of the present invention produced in this manner typically have cohesive termini, i.e., "overhanging" single-stranded portions that extend beyond the double-stranded portion of the molecule. The presence of cohesive termini on the DNA molecules of the present invention is preferred.

Also contemplated by the present invention are ribonucleic acid (RNA) equivalents of the above described DNA molecules.

The present invention further contemplates a recombinant DNA molecule comprising a vector operatively linked, for replication and/or expression, to a subject DNA molecule, i.e., a DNA molecule defining a gene coding for a subject polypeptide or a subject chimeric polypeptide.

As used herein, the term "vector" refers to a DNA molecule capable of autonomous replication in a cell and to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of a gene delivered by a subject DNA segment are referred to herein as "expression vectors". Thus, a recombinant DNA molecule (rDNA) is a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature.

The choice of vector to which a DNA segment of the present invention is operatively linked depends directly, as is well known in the art, on the functional properties desired, e.g., protein expression, and the host cell to be transformed, these

being limitations inherent in th art of c nstructing recombinant DNA molecules. However, a vector contemplated by the present invention is at least capable of directing the replication, and preferably also expression, of the subject chimeric polypeptide gene included in DNA segments to which it is operatively linked.

In preferred embodiments, a vector contemplated by the present invention includes a procaryotic replicon, i.e., a DNA sequence having the ability to direct autonomous replication and maintenance of the recombinant DNA molecule extrachromosomally in a procaryotic host cell, such as a bacterial host cell, transformed therewith. Such replicons are well known in the art. In addition, those embodiments that include a procaryotic replicon also include a gene whose expression confers drug resistance to a bacterial host transformed therewith. Typical bacterial drug resistance genes are those that confer resistance to ampicillin or tetracycline.

Those vectors that include a procaryotic replicon can also include a procaryotic promoter capable of directing the expression (transcription and translation) of the subject chimeric polypeptide gene in a bacterial host cell, such as E. coli, transformed therewith. A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention. Typical of such vector plasmids are pUC8, pUC9, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA) and pPL and pKK223 available from Pharmacia, Piscataway, N.J.

Expression vectors compatible with eucaryotic cells, preferably those compatible with vertebrate cells, can also be used to form the recombinant DNA molecules of the present invention. Eucaryotic cell expression vectors are well known in the art and are available from several commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired DNA segment. Typical of such vectors are pSVL and pKSV-10 (Pharmacia), pBPV-1pML2d (International Biotechnologies, Inc.), and pTDT1 (ATCC, #31255).

In preferred embodiments, the eucaryotic cell expression vectors used to construct the recombinant DNA molecules of the present invention include a selection marker that is effective in an eucaryotic cell, preferably a drug resistance selection marker. A preferred drug resistance marker is the gene whose expression results in neomycin resistance, i.e., the neomycin phosphotransferase (neo) gene. Southern et al., J. Mol. Appl. Genet., 1:327-341 (1982).

The use of retroviral expression vectors to form the rDNA of the present invention is also contemplated. As used herein, the term "retroviral expression vector" refers to a DNA molecule that includes a promoter sequence derived from the long terminal repeat (LTR) region of a retrovirus genome.

In preferred embodiments, the expression vector is typically a retroviral expression vector that is preferably replication-incompetent in eucaryotic cells. The construction and use of retroviral vectors has been described by Sorge, et al., Mol. Cell. Biol., 4:1730-37 (1984).

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instanc , complem ntary

homopolymer tracts can b added to the DNA segment to be inserted and to the v ctor DNA. The vect r and DNA segment are then joined by hydrogen bonding between the complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase of E. coli DNA polymerase I, enzymes that remove protruding, 3', single-stranded termini with their 3'-5' exonucleotytic activities and fill in recessed 3' ends with their polymerizing activities. The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies, Inc., New Haven, CN.

Also contemplated by the present invention are RNA equivalents of the above described recombinant DNA

molecules.

The present invention also relates to a host cell transformed with a recombinant DNA molecule of the present invention preferably an rDNA capable of expressing a subject chimeric polypeptide. The host cell can be either procaryotic or eucaryotic. Bacterial cells are preferred procaryotic host cells and typically are a strain of E. coli such as, for example, the E. coli strain DH5 available from Bethesda Research Laboratories, Inc., Bethesda, MD. Preferred eucaryotic host cells include yeast and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Preferred eucaryotic host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61 and NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658. Transformation of appropriate cell hosts with a recombinant DNA molecule of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of procaryotic host cells, see, for example, Cohen et al., Proc. Natl. Acad. Sci. USA, 69:2110 (1972); and Maniatis et al., Molecular Cloning, A Laboratory Mammal, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982). With regard to transformation of vertebrate cells with retroviral vectors containing rDNAs, see, for example, Sorge et al., Mol. Cell. Biol., 4:1730-37 (1984); Graham et al., Virol., 52:456 (1973); and Wigler et al., Proc. Natl. Acad. Sci. USA, 76:1373-76 (1979).

Successfully transformed cells, i.e., cells that contain a recombinant DNA molecule of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an rDNA of the present invention can be cloned to

produce monoclonal colonies. Cells from those coloni s can be harvested, lysed and their DNA content examined for the presence of the rDNA using a method such as that described by Southern, J. Mol. Biol., 98-503 (1975) or Berent et al., Biotech., 3:208 (1985).

In addition to directly assaying for the presence of rDNA, successful transformation can be confirmed by well known immunological methods when the rDNA is capable of directing the expression of a subject chimeric polypeptide. For example, cells successfully transformed with an expression vector produce proteins displaying tenascin receptor-binding region antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the presence of tenascin receptor-binding region antigenicity using antipolypeptide antibodies specific for that region.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium. Preferably, the culture also contains a protein displaying tenascin receptor-binding activity.

Nutrient media useful for culturing transformed host cells are well known in the art and can be obtained from several commercial sources. In embodiments wherein the host cell is mammalian, a "serum-free" medium is preferably used.

E. Inocula

In another embodiment, a polypeptide of this invention, preferably a peptide corresponding to formula p1 or p2 is used in a pharmaceutically acceptable aqueous diluent composition to form an

inoculum that, when administered in an effective amount, is capable of inducing antibodies that immunoreact with tenascin and inhibit its ability to facilitate cell attachment.

The word "inoculum" in its various grammatical forms is used herein to describe a composition containing a polypeptide of this invention as an active ingredient used for the preparation of antibodies against an Integrin beta subunit.

When a polypeptide is used to induce antibodies it is to be understood that the polypeptide can be used alone, or linked to a carrier as a conjugate, or as a polypeptide polymer, but for ease of expression the various embodiments of the polypeptides of this invention are collectively referred to herein by the term "polypeptide", and its various grammatical forms.

As already noted, one or more additional amino acid residues can be added to the amino- or carboxy-termini of the polypeptide to assist in binding the polypeptide to a carrier. Cysteine residues added at the amino- or carboxy-termini of the polypeptide have been found to be particularly useful for forming conjugates via disulfide bonds. However, other methods well known in the art for preparing conjugates can also be used. Exemplary additional linking procedures include the use of Michael addition reaction products, di-aldehydes such as glutaraldehyde, Klipstein et al., J. Infect. Dis., 147, 318-326 (1983) and the like, or the use of carbodimide technology as in the use of a water-soluble carbodimide to form amide links to the carrier. For a review of protein conjugation or coupling through activated functional groups, see Aurameas, et al., Scand. J. Immunol., Vol. 8, Suppl. 7:7-23 (1978).

Useful carriers are well known in the art, and are generally proteins themselves. Exemplary of such carriers are keyhole limpet hemocyanin (KLH), edestin, thyroglobulin, albumins such as bovine serum albumin (BSA) or human serum albumin (HSA), red blood cells such as sheep erythrocytes (SRBC), tetanus toxoid, cholera toxoid as well as polyamino acids such as poly (D-lysine: D-glutamic acid), and the like.

The choice of carrier is more dependent upon the ultimate use of the inoculum and is based upon criteria not particularly involved in the present invention. For example, a carrier that does not generate an untoward reaction in the particular animal to be inoculated should be selected.

The present inoculum contains an effective, immunogenic amount of a polypeptide of this invention, typically as a conjugate linked to a carrier. The effective amount of polypeptide or protein per unit dose depends, among other things, on the species of animal inoculated, the body weight of the animal and the chosen inoculation regimen as is well known in the art. Inocula typically contain polypeptide or protein concentrations of about 10 micrograms to about 500 milligrams per inoculation (dose), preferably about 50 micrograms to about 50 milligrams per dose.

The term "unit dose" as it pertains to the inocula of the present invention refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to produce the desired immunogenic effect in association with the required diluent; i.e., carrier, or vehicle. The specifications for the novel unit dose of an inoculum of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the

particular immunologic effect to be achi ved, and (b) the limitations inherent in the art of compounding such active material for immunologic use in animals, as disclosed in detail herein, these being features of the present invention.

Inocula are typically prepared from the dried solid polypeptide-conjugate by dispersing the polypeptide-conjugate in a physiologically tolerable (acceptable) diluent or vehicle such as water, saline or phosphate-buffered saline to form an aqueous composition. Such diluents are well known in the art and are discussed, for example, in Remington's Pharmaceutical Sciences, 16th Ed., Mack Publishing Company, Easton, PA (1980) at pages 1465-1467.

Inocula can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

F. Polyclonal and Monoclonal Anti-peptide Antibodies

An antibody of the present invention, whether polyclonal or monoclonal, immunorreacts with tenascin and a peptide according to formula p1. The antibody inhibits endothelial cell attachment. The antibody does not immunoreact with a peptide represented by the formula:

NH_2-Gly-Arg-Gly-Asp-Ser-Pro-COOH,

and does not block the attachment of endothelial cells to either fibronectin or laminin.

The term "antibody" in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules

that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', $F(ab')_2$ and F(v).

An "antibody combining site" is that structural portion of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) antigen. The term "immunoreact" in its various forms means binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

"Antigenic determinant" refers to the actual structural portion of the antigen that immunologically bound by an antibody combining site. The terms is also used interchangeably with "epitope".

1. Polyclonal Antibodies

A polyclonal antibody of the present invention immunoreacts with a subject polypeptide, preferably a polypeptide corresponding in amino acid residue sequence to the formula p1. A subject polyclonal antibody is further characterized as not substantially immunoreacting with a polypeptide having an amino acid residue sequence of the formula: Gly-Arg-Gly-Asp-Ser-Pro.

A preferred polyclonal antibody is characterized as having the ability to immunoreact with tenascin and inhibit the capacity of tenascin promote cell attachment, tumor metastasis, angiogenesis and the like.

A polyclonal antibody of the present invention is typically produced by immunizing a mammal with an inoculum of the present invention, preferably an inoculum containing a peptide corresponding to a Formula I and thereby induce in the mammal antibody molecules having the appropriate polypeptide immunospecificity. The antibody molecules are then collected from the mammal and isolated to the extent desired by well known techniques such as, for example, by immunoaffinity chromatography using the immunizing polypeptide in the solid phase. The polyclonal antibody so produced can be used in, inter alia, the diagnostic methods and systems of the present invention to discriminate between activated and nonactivated platelets or nucleated cells and in therapeutic methods for the purpose of modulating cell adhesion, such as inhibiting platelet adhesion.

2. Monoclonal Antibodies

A monoclonal antibody of the present invention is characterized as immunoreacting with an epitope formed by the amino acid residue sequence Ser-Arg-Arg-Gly-Asp-Met-Ser. Preferably, a subject monoclonal antibody is further characterized as not immunoreacting with a polypeptide corresponding to the amino acid residue sequence Gly-Arg-Gly-Asp-Ser-Pro.

A preferred monoclonal antibody is also characterized as having the ability to inhibit the specific binding between tenascin and its receptor, and thereby inhibit cell adhesion, metastasis, angiogenesis and the like.

The phrase "monoclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with a

particular antigen. A monoclonal antibody composition thus typically displays a single binding affinity f r any antigen with which it immunoreacts. A monoclonal antibody composition may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen, e.g., a bispecific monoclonal antibody.

A monoclonal antibody is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) but one kind of antibody molecule. The hybridoma cell is formed by fusing an antibody-producing cell and a myeloma or other self-perpetuating cell line. Such antibodies were first described by Kohler and Milstein, Nature 256:495-497 (1975), which description is incorporated by reference.

3. Methods for Producing A Monoclonal Antibody

The present invention contemplates a method of forming a monoclonal antibody that immunoreacts with a polypeptide of formula p1, but does not immunoreact with a peptide of the formula Gly-Arg-Asp-Gly-Ser-Pro. The method comprises the steps of:

(a) Immunizing an animal with a tenascin or a subject polypeptide, preferably a peptide according to formula p1. This is typically accomplished by administering an immunologically effective amount i.e., an amount sufficient to produce an immune response, of the immunogen to an immunologically competent mammal. Preferably, the mammal is a rodent such as a rabbit, rat or mouse. The mammal is then maintained for a time period sufficient for the mammal to produce cells secreting antibody molecules that immunoreact with the immunogen.

(b) A suspension of antibody-producing cells removed from th immunized mammal is then prepared. This is typically accomplished by removing the spleen of the mammal and mechanically separating the individual spleen cells is a physiologically tolerable medium using methods well known in the art.

(c) The suspended antibody producing cells are treated with a transforming agent capable of producing a transformed ("immortalized") cell line. Transforming agents and their use to produce immortalized cell lines are well known in the art and include DNA viruses such as Epstein Bar Virus (EBV), Simian Virus 40 (SV40), Polyoma Virus and the like, RNA viruses such as Moloney Murine Leukemia Virus (Mo-MuLV), Rous Sarcoma Virus and the like, myeloma cells such as P3X63-Ag8.653, Sp2/O-Ag14 and the like.

In preferred embodiments, treatment with the transforming agent results in the production of a hybridoma by fusing the suspended spleen cells with mouse myeloma cells from a suitable cell line by the use of a suitable fusion promoter. The preferred ratio is about 5 spleen cells per myeloma cell. A total volume of about 10^8 splenocytes.

The cell line used should preferably be of the so-called "drug resistant" type, so that unfused myeloma cells will not survive in a selective medium, while hybrids will survive. The most common class is 8-azaguanine resistant cell lines, which lack the enzyme hypoxanthine guanine phophoribosyl transferase and hence will not be supported by HAT (hypoxanthine, aminopterin, and thymidine) medium. It is also generally preferred that the myeloma cell line used be of the so-called "non-secreting" type, in that it does not itself produce any antibody, although secreting types may be used. In certain cases, however,

secreting myeloma lin s may be preferred. While the preferred fusion promoter is polyethylene glycol having an average molecule weight from about 1000 to about 4000 (commercially available as PEG 1000, etc.), other fusion promoters known in the art maybe employed.

(d) The transformed cells are then cloned, preferably to monoclonality. The cloning is preferably performed in a tissue culture medium that will not support non-transformed cells. When the transformed cells are hybridomas, this is typically performed by diluting and culturing in separate containers, the mixture of unfused spleen cells, unfused myeloma cells, and fused cells (hybridomas) in a selective medium which will not support the unfused myeloma cells for a time sufficient to allow death of the unfused cells (about one week). The dilution may be a type of limiting one, in which the volume of diluent is statistically calculated to isolate a certain number of cells (e.g., 1-4) in each separate container (e.g., each well of a microtiter plate). The medium is one (e.g., HAT medium) which will not support the drug-resistant (e.g., 8-azaguanine resistant) unfused myeloma cell line.

(e) The tissue culture medium of the cloned transformants is evaluated for the presence of secreted antibody molecules that immunoreact with tenascin and a polypeptide according to formula p1. Peptide positive transformants are preferably further screened to identify those that do not react with the peptide Gly-Arg-Asp-Gly-Ser-Pro.

(f) Once a desired transformant has been identified in step (e), it is selected and grown in a suitable tissue culture medium for a suitable length of time, followed by recovery of the desired antibody from the culture supernatant. The suitable medium and

suitable length of culturing time are known or are readily determined.

To produce a much greater concentration of slightly less pure monoclonal antibody, the desired hybridoma may be injected into mice, preferably syngenic or semisyngenic mice. The hybridoma will cause formation of antibody-producing tumors after a suitable incubation time, which will result in a high concentration of the desired antibody (about 5-20 mg/ml) in the bloodstream and peritoneal exudate (ascites) of the host mouse.

Media useful for the preparation of these compositions are both well known in the art and commercially available and include synthetic culture media, inbred mice and the like. An exemplary synthetic medium is Dulbecco's minimal essential medium [DMEM; Dulbecco et al., Virol. 8:396 (1959)] supplemented with 4.5 gm/1 glucose, 20 mm glutamine, and 20% fetal calf serum. An exemplary inbred mouse strain is the Balb/c.

A monoclonal antibody of the present invention can also be further purified by well known immunoaffinity chromatography methods by using in the solid phase a subject polypeptide with which the antibody immunoreacts.

A monoclonal antibody produced by the above method can be used, for example, in diagnostic and therapeutic modalities wherein formation of a tenascin immunoreaction product is desired.

G. Hybridomas and Methods of Preparation

Hybridomas of the present invention are those which are characterized as having the capacity to produce a subject monoclonal antibody.

Meth ds for producing hybridomas producing (secreting) antibody molecules having a desired immunospecificity, i.e., having the ability to immunoreact with a particular protein, an identifiable epitope on a particular protein and/or a polypeptide, are well known in the art. Particularly applicable is the hybridoma technology described by Niman et al., Proc. Natl. Acad. Sci. USA, 80:4949-4953 (1983), and by Galfre et al., Meth. Enzymol., 73:3-46 (1981), which descriptions are incorporated herein by reference.

H. Therapeutic Methods and Compositions

A subject polypeptide can be used to modulate the attachment in vivo of cells expressing the tenascin receptor, e.g., endothelial cells and undifferentiated tumor cells.

For instance, a subject polypeptide, preferably a peptide corresponding to formula p1 or p2, can be used in a pharmaceutically acceptable composition that, when administered to a human subject in an effective amount, is capable of competitively inhibiting cell attachment to the subcellular matrix. That inhibition is believed to result in a decreased rate of tumor formation.

In another embodiment, the attachment of cells can be inhibited by intravenous and/or subcutaneous administration of an effective amount of a pharmaceutically acceptable composition comprising a subject polyclonal antibody that immunoreacts with a polypeptide corresponding to formula p1 or p2.

A preferred method of modulating cell adhesion contemplates administering a cell attachment-inhibiting amount of a subject monoclonal antibody that immunoreacts with a polypeptide corresponding to formula p1.

The polypeptide- or antibody molecule-containing compositions administered take the form of solutions or suspensions, however, polypeptides can also take the form of tablets, pills, capsules, sustained release formulations or powders. In any case, the polypeptide-containing compositions typically contain about 0.1 uM to about 1.0 M of polypeptide as active ingredient, preferably about 1.0 uM to about 10 millimolar (mM), whereas the antibody molecule-containing compositions typically contain about 10 ug/ml to about 20 mg/ml of antibody as active ingredient, preferably about 1 mg/ml to about 10 mg/ml.

The preparation of a therapeutic composition that contains polypeptides or antibody molecules as active ingredients is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient as are well known. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

A polypeptide or antibody can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of

the polypeptide or antibody molecule) that ar formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic polypeptide- or antibody containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosages for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent, i.e., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject to utilize the active ingredient, and degree of inhibition of receptor-ligand binding desired. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of one to several milligrams of active ingredient per individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by

r peated doses at one or more hour intervals by a subsequent injection or th r administration. Alternatively, continuous intravenous infusion sufficient to maintain therapeutically effective concentrations in the blood are contemplated. For a subject polypeptide, therapeutically effective blood concentrations are in the range of about 0.1 mM to about 10 mM, preferably about 1.0 mM. Therapeutically effective blood concentrations of antibody molecules of the present invention are in the range of about 0.1 uM to about 10 uM, preferably 1.0 uM.

I. Diagnostic Systems

A diagnostic system in kit form of the present invention includes, in an amount sufficient for at least one assay, a polypeptide, polyclonal antibody or monoclonal antibody of the present invention, as a separately packaged reagent. Instructions for use of the packaged reagent are also typically included.

"Instructions for use" typically include a tangible expression describing the reagent concentration or at least one assay method parameter such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions and the like.

In one embodiment, a diagnostic system for assaying for tumor cells in a sample, such as a tumor biopsy, comprises a package containing a subject antibody that immunoreacts with a polypeptide corresponding to formula p1. In another embodiment, a diagnostic system for assaying for tumor cells in a sample comprises a package containing a subject monoclonal antibody. Further preferred are kits wherein the antibody molecules of the polyclonal or

monoclonal antibody are linked to a label.

Thus, in preferred embodiments, a diagnostic system of the present invention further includes a label or indicating means capable of signaling the formation of a complex containing the antibody molecules of a polyclonal or monoclonal antibody of the present invention.

The word "complex" as used herein refers to the product of a specific binding reaction such as an antibody-antigen or receptor-ligand reaction. Exemplary complexes are immunoreaction products.

As used herein, the terms "label" and "indicating means" in their various grammatical forms refer to single atoms and molecules that are either directly or indirectly involved in the production of a detectable signal to indicate the presence of a complex. "In vivo" labels or indicating means are those useful within the body of a human subject and include ^{111}In, ^{99}Tc, ^{67}Ga, ^{186}Re, and ^{132}I. Any label or indicating means can be linked to or incorporated in an expressed protein, polypeptide, or antibody molecule that is part of an antibody or monoclonal antibody composition of the present invention, or used separately, and those atoms or molecules can be used alone or in conjunction with additional reagents. Such labels are themselves well-known in clinical diagnostic chemistry and constitute a part of this invention only insofar as they are utilized with otherwise novel protein methods and/or systems.

Preferred labels include alkaline phosphatase [O'Sullivan et al., FEBS Letters, 95:311 (1978)], biotin, horse radish peroxidase, dichlorotriazinyl-aminofluorescein [DTAF; Blakeslee et al., J. Immunol. Meth., 13:320 (1977)], ferritin [Carlsson et al, Biochem. J., 173:723 (1978)], fluoroscene

isothiocyanste [FITC; McKinney et al., Anal. Biochem, 14:421 (1966)], beta-galactosidase [Ishikawa et al., Scand. J. Immunol., 8:43 (1978)], sulforhodamine 101 acid chloride (Texas Red), tetramethyrhodamine isothiocyanate [TRITC; Amante et al., J. Immunol. Meth., 1:289 (1972)], gold [Horisberger et al., Histochem., 82:219 (1985)], and the like.

The linking of labels, i.e., labeling of, polypeptides and proteins is well known in the art. For instance, antibody molecules produced by a hybridoma can be labeled by metabolic incorporation of radioisotope-containing amino acids provided as a component in the culture medium. See, for example, Galfre et al., Meth. Enzymol., 73:3-46 (1981). The techniques of protein conjugation or coupling through activated functional groups are particularly applicable. See, for example, Aurameas, et al., Scand. J. Immunol., Vol. 8 Suppl. 7:7-23 (1978), Rodwell et al., Biotech., 3:889-894 (1984), and U.S. Pat. No. 4,493,795.

The diagnostic systems can also include, preferably as a separate package, a specific binding agent. A "specific binding agent" is a molecular entity capable of selectively binding a reagent species of the present invention but is not itself a protein expression product, polypeptide, or antibody molecule of the present invention. Exemplary specific binding agents are antibody molecules, complement proteins or fragments thereof, protein A and the like. Preferably, the specific binding agent can bind the antibody molecule or polypeptide of this invention when it is present as part of a complex.

In preferred embodiments the specific binding agent is labeled. However, when the diagnostic system includes a sp cific binding agent that is not labeled,

the agent is typically used as an amplifying means or reagent. In these embodiments, the labeled specific binding agent is capable of specifically binding the amplifying means when the amplifying means is bound to a reagent species-containing complex.

The diagnostic kits of the present invention can be used in an "ELISA" format to detect the presence or quantity of fibrinogen-bound platelets in a body fluid sample such as serum, plasma or urine. "ELISA" refers to an enzyme-linked immunosorbent assay that employs an antibody or antigen bound to a solid phase and an enzyme-antigen or enzyme-antibody conjugate to detect and quantify the amount of an antigen or antibody present in a sample. A description of the ELISA technique is found in Chapter 22 of the 4th Edition of Basic and Clinical Immunology by D.P. Sites et al., published by Lange Medical Publications of Los Altos, CA in 1982 and in U.S. Patents No. 3,654,090; No. 3,850,752; and No. 4,016,043, which are all incorporated herein by reference.

Thus, in preferred embodiments, the expressed protein, polypeptide, or antibody molecule of the present invention can be affixed to a solid matrix to form a solid support that is separately packaged in the subject diagnostic systems.

The reagent is typically affixed to the solid matrix by adsorption from an aqueous medium although other modes of affixation, well known to those skilled in the art can be used.

Useful solid matrices are well known in the art. Such materials include the cross-linked dextran available under the trademark SEPHADEX from Pharmacia Fine Chemicals (Piscataway, NJ); agarose; beads of polystyrene beads about 1 micron to about 5 millimeters in diameter available from Abbott Laboratories of North

Chicago, IL; polyvinyl chloride, polystyr ne, cr ss-linked polyacrylamide, nitroc llul se- r nylon-bas d webs such as sheets, strips or paddles; or tubes, plates or the wells of a microtiter plate such as those made from polystyrene or polyvinylchloride.

The reagent species, labeled specific binding agent or amplifying reagent of any diagnostic system described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. Where the indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support such as the before-described microtiter plate and one or more buffers can also be included as separately packaged elements in this diagnostic assay system.

The packages discussed herein in relation to diagnostic systems are those customarily utilized in diagnostic systems. Such packages include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, plastic and plastic-foil laminated envelopes and the like.

J. Assay Methods

The present invention contemplates any method that results in detecting tenascin, by producing a complex containing an antibody molecule contained in a polyclonal antibody or monoclonal antibody of the present invention. Those skilled in the art will understand that there are numerous well known clinical diagnostic chemistry procedures that can be utilized to form those complexes. Thus, while exemplary assay methods are described herein, the invention is not so limited.

For example, a tumor bi psy sample and ^{125}I-

labeled antibody mol cules are admixed. The immunoreaction admixture thus formed is maintained under immunological assay conditions for a time period sufficient for any undifferentiated tumor cells expressing tenascin to immunoreact with the labeled antibodies and form a labeled immunoreaction product. The labeled immunoreaction products are then separated from the non-reacted labeled-antibodies, typically by centrifugation sufficient to pellet all cells present in the sample. The amount of labeled immunoreaction product formed is then assayed.

Immunological assay conditions are those that maintain the immunological activity of the antibody molecules contained in a polyclonal or monoclonal antibody of this invention and the Integrin molecules sought to be assayed. Those conditions include a temperature range of about 4 degrees C to about 45 degrees C, preferably about 37 degrees C, a pH value range of about 5 to about 9, preferably about 7 and an ionic strength varying from that of distilled water to that of about one molar sodium chloride, preferably about that of physiological saline. Methods for optimizing such conditions are well known in the art.

In another embodiment, an aliquot of an antibody of this invention having antibody molecules linked to an <u>in vivo</u> label are intravenously administered to a subject. After a predetermined period of time sufficient for the antibody molecules to immunoreact, the subject is then assayed for the presence of tenascin-containing labeled immunoreaction products.

<u>Examples</u>

The following examples illustrate but do not limit the present invention.

1. Peptide Synthesis

P ptides used in cell attachment assays were assayed for purity by HPLC and for cytotoxicity. The peptides SRRGDMS-NH_2, MGSRSRD-NH_2, GRGDSP-NH_2 and LLGAKQAGDV-NH_2, purchased from Multiple Peptide Systems, La Jolla, CA, were synthesized with a carboxy terminal amine because that has been shown to dramatically affect RGD peptide activity. Pierschbacher et al., J. Biol. Chem., 262:17294-17298, (1987). The peptide, GRGDESP, was provided by Pena Cardarelli, Immunetech Pharmaceuticals, San Diego, CA, and the peptide SDDYSGSGSG was synthesized as previously described, Bourdon et al., Proc. Natl. Acad. Sci. USA, 84:3194-3198, (1987). Conjugates of peptide were prepared by mixing 1 mg each peptide and keyhole limpet homocyamin (KLH) or bovine serum albumin (BSA), adding glutaraldehyde (.025 M) and further mixing for 3 hours at room temperature. Conjugates were then concentrated and buffer exchanged into phosphate buffered saline (PBS) using Centricon 10 concentrators (Amicon).

2. Tenascin Purification

Tenascin was isolated from the spent culture media of U251 human glioma cells. Culture media was first concentrated by salt precipitation of proteins with 40% ammonium sulfate. The precipitated material was then resolubilized in a small volume of PBS, containing a 1% Triton-X 100, 2 M area and size chromatographed over a 2.5 cm X 120 cm Sephacryl-500 column. Fractions corresponding to tenascin as determined by SDS-PAGE and immunoblot were pooled. Pooled fractions were then further purified by DEAE anion exchange chromatography and finally 81C6 anti-tenascin monoclonal antibody affinity chromatography.

The resulting tenascin is greater than 99% pure with yields of appr ximately 70%.

3. Cell Attachment Studies

The human cell line, human glioma U251, was grown in DMEM (GIBCO Laboratories, Grand Island, NY) containing 10% FBS (Tissue Culture Biologicals, Tulare, CA) and gentamicin. Cultures were maintained in a humidified 7% CO_2 atmosphere at 37°C.

Cell attachment assays were performed with cells harvested from cultures in log phase growth. Typically, cells were treated with DiI, a fluorescent dye, (40 µg/ml) (Molecular Probes) for 1 hour prior to cell harvest. Cells were harvested using 0.02 M EDTA in PBS to detach the cells. The harvested cells were centrifuged to form a pellet of cells. The resultant pellet of cells was resuspended in DMEM - 1% BSA and aliquoted into microtiter wells (3 x 10^5 cells/ml), to which either peptides or antibodies in DMEM - 1% BSA were separately admixed to form either a cell-peptide admixture or cell antibody admixture. The admixtures were aliquoted to individual wells of Falcon pro-bind plates. The tissue culture plates containing ells in the absence of presence of peptide or antibody were then maintained for 1 to 1.5 hours at 37°C. The plates were subjected to cytofluorometric analysis to determine the amount of fluorescent dye, thereby obtaining a relative indication of cell attachment. For cell attachment assays described below, the individual wells were coated with protein or protein-peptide conjugates at various concentrations for 4 to 16 hours prior to the addition of cells.

A. Attachment to Tenascin

Tenascin, prepared in Exampl 2, was diluted in PBS to a concentration of 3 μg/ml. Fibronectin and laminin, which are commercially available, were similarly dissolved and diluted to a concentration of 3 μg/ml. Coated plates were then washed and incubated for 30 minutes with a solution containing the DMEM-BSA medium to block nonspecific binding sites before their use in cell attachment assays. The cell attachment assay was then performed as described above.

The results of this assay are shown in Figure 2. Cells attached to all three extracellular matrix substrates, tenascin, fibronectin and laminin, with similar profiles saturating the plate with a fluorescence reading of 1.0.

B. Attachment to Tenascin Peptide SRRGDMS-BSA

In order to directly examine whether the tenascin peptide SRRGDMS-NH_2 mediated binding of tenascin-specific receptors on U251 cells, the peptide was conjugated to BSA using glutaraldehyde and the SRRGDMS-BSA peptide was then used to coat wells of a microtiter plate. Cell attachment assays were performed as described above.

The results, shown in Figure 3, revealed that SRRGDMS-BSA supports cell attachment. Titration with increasing amounts of SRRGDMS-BSA coating resulted in increasing levels of cell attachment to a maximum at a coating concentration equivalent to 2.5 μg/ml BSA (closed squares). Half-maximal attachment on SRRGDMS-BSA was seen at the concentration of 0.5 μg/ml BSA (closed triangles). Conjugates of GRGDSP-BSA gave similar results while the MGSRSRD-BSA conjugate control (scrambled tenascin-derived peptide) did not support

cell attachment (open circles).

4. Inhibition of Tenascin Mediated Cell Attachment by Soluble SRRGDMS-NH_2 Peptide

A. Substrate Specificity

To test whether the RGD-containing region of tenascin was a cell attachment site, the peptide SRRGDMS-NH_2 from human tenascin was examined in cell attachment inhibition assays. The assays were performed as described for cell attachment assays in Example 3 on the extracellular matrix substrates, tenascin (TN), plasma fibronectin (FN) and laminin (LM). The attachment of cells to the different substrates was measured in the presence of media or synthetic peptides admixed at a final concentration of 1 mg/ml. The synthetic peptides evaluated in the assay for their ability to inhibit the interaction of cells with the substrate included the following: 1) SRRGDMS (tenascin-derived); 2) GRGDSP (fibronectin cell attachment signal-derived); 3) GRGESP (negative control); and 4) SDDYSGSGSG (glycosaminoglycan attachment signal of serglycin-derived).

The results of the inhibition of cell attachment assays are shown in Figure 4. Cell attachment to tenascin was inhibited by the tenascin derived peptide SRRGDMS-NH_2 but not by the serglycin-derived peptide, SDDYSGSGSG, nor by the peptide GRGESP. In addition to inhibiting cell attachment to tenascin, the SRRGDMS-NH_2 peptide also inhibited cell attachment to fibronectin but not to laminin. The fibronectin derived peptide, GRGDSP, exhibited the same pattern as SRRGDMS-NH_2. Tenascin peptides containing the RGD sequence are, therefore, active in competing with tenascin for receptor binding sites.

B. Titration Curves

The activity of th SRRGDMS-NH_2 peptide was further assayed by titrating peptide inhibition of cell attachment to tenascin and fibronectin. The assays were performed as described in Example 3 and 4 with the concentration of the SRRGDMS-NH_2 peptide ranging in from 1 μM up to 40 mM.

Titration curves demonstrated that SRRGDMS-NH_2 inhibited cell attachment in a dose dependent manner as shown in Figure 5. The peptide concentration resulting in half-maximal inhibition of cell attachment to tenascin was in the range of 40 to 70 μM. This contrasts with the 2 to 4 mM concentrations of peptide needed for half-maximal cell attachment inhibition on fibronectin. Comparison of the SRRGDMS-NH_2 titration curves on tenascin and fibronectin indicate a much greater sensitivity to peptide inhibition on tenascin than fibronectin. These results show that levels of SRRGDMS peptide which can completely inhibit cell attachment to tenascin do not effect fibronectin mediated cell attachment.

5. Inhibition of Tenascin Peptide-BSA Mediated Cell Attachment by Soluble SRRGDMS-NH_2 Peptide

Both of the synthetic peptides, SRRGDMS and GRGDSP, conjugated to BSA, promoted cell attachment as described in Example 3 and shown in Figure 3. The effect of admixed soluble synthetic peptides on cell attachment to peptide-coated wells was determined in inhibition assays performed as described in Example 4. Individual wells of the microtiter plates were coated with either 1 μg/ml of SRRGDMS-BSA or 0.5 μg/ml of GRGDSP-BSA. Attachment of U251 cells to the peptide-coated wells was assayed either in the absence of peptid , for measuring maximum cell attachm nt, r in

the presence of 1 mg/ml of the following synthetic peptides: SRRGDMS-NH_2; GRGDSP-NH_2; or MGSRSRD-NH_2.

The results of the inhibition of cell attachment assays on peptide-coated plates by soluble peptides are shown in Figure 6. The cell attachment mediated by SRRGDMS-BSA was inhibited by both SRRGDMS-NH_2 and GRGDSP-NH_2 but not by the MGSRSRD-NH_2 control peptide when compared to untreated cells. The results demonstrate that the peptide is recognized by cell attachment receptors on cell surfaces with characteristic RGD dependent cell attachment as observed with tenascin.

6. Preparation and Characterization of Polyclonal Antibodies Directed Against Tenascin and Tenascin-Derived Peptide SRRGDMS-Coupled to KLH

A. Preparation of Polyclonal Antibodies

Antibodies to tenascin were prepared by immunization of rabbits with purified human tenascin in MPL - TDM adjuvant (RIBI Immunochem Research, Hamilton, MT). Rabbit polyclonal antibodies were raised against SRRGDMS-NH_2 coupled to KLH using glutaraldehyde.

Peptide - KLH conjugates in adjuvant were used to immunize rabbits for preparation of anti peptide. Immunoglobin fractions of antisera were prepared by sodium sulfate precipitation and resuspension in PBS. Antibodies were affinity purified on purified human tenascin-sepharose.

B. Characterization of Polyclonal Antibodies

An IgG fraction of the anti peptide antibodies was prepared and examined in ELISA for reactivity to SRRGDMS-NH_2 and control peptide MGSRSRD-NH_2 and other peptides coupled to BSA. The anti-SRRGDMS antibody at a concentration of 1mg/ml IgG was

admixed into microtiter wells previously coat d with separate s lutions of above synthetic peptides at a final concentration of 1 μg/ml to form an immunoreaction admixture.

The microtiter plate was maintained for one hour to result in an immunoreacted product. The maintenance period was followed by washing with PBS containing 0.02% Tween 20. The immunoreactant products were detected by admixing a solution of goat anti-rabbit alkaline phosphatase conjugated secondary antibodies into each well to form a second immunoreactant admixture. The resultant immunoreaction was detected by the chromogenic substrate, PNPP (Signa Chemicals, St. Louis, MO). The optical density of the microtiter plate was measured at an absorbance of 450 nm.

The results of the ELISA, shown in Figure 7, revealed that the SRRGDMS-NH_2 antibodies bound only to SRRGDMS-BSA indicating that the antibodies were specific for SRRGDMS-NH_2. The antibodies did not immunoreact with the other RGD-containing peptide, GRGDSP-NH_2. This result indicates that the peptides represent distinct epitopes.

7. Inhibition of Tenascin and SRRGDMS-BSA Mediated Cell Attachment by Anti-Tenascin Antibodies and Affinity Purified Anti-SRRGDMS Antisera

A. Inhibition of Tenascin Mediated Cell Attachment

The functional importance of the SRRGDMS sequence at the cell attachment site of tenascin was also approached using antibodies to SRRGDMS-NH_2. The antibodies to the SRRGDMS-NH_2 peptide were used in inhibition of cell attachment assays to determine if they would bind tenascin and thereby, inhibit cell

attachment.

The assays were performed as described in Example 4 with the exception that antibodies to either tenascin or SRRGDMS were used in place of soluble peptides. Either 100 μg anti-tenascin antibody or 50 μg anti-SRRGDMS antibody affinity purified on tenascin was used in the assay.

The results of the inhibition of cell attachment assays on the separate extracellular matrix substrates, tenascin, fibronectin and laminin, by anti-tenascin or anti-peptide antibodies are shown in Figure 2. Both antibodies were effective at inhibiting cell attachment to tenascin but not fibronectin, laminin or BSA. The results demonstrate that the anti SRRGDMS antibodies not only recognize tenascin but bind at the cell attachment site.

B. Inhibition of Tenascin Peptide SRRGDMS-BSA Mediated Cell Attachment

The antibodies described in A. above were used in inhibition of cell attachment assays to determine if they could bind to specific synthetic peptides and thereby inhibit cell attachment. The assays were performed as described in Example 5 with the exception that antibodies were used in place of soluble synthetic peptides. Microtiter wells were coated with either 1 μg/ml SRRGDMS-BSA or 0.5 μg/ml GRGDSP-BSA as described in Example 1. In this assay, of either 50 μg of the anti-tenascin antibody affinity purified on SRRGDMS-sepharose or 50 μg of the anti-SRRGDMS antibody were used.

In the above inhibition assays, both anti-tenascin and the anti-SRRGDMS antibodies inhibited cell attachment to SRRGDMS-BSA but not cell attachment to GRGDSP-BSA. The results, shown in Figure 8, in

addition to thos shown in Figure 7, confirm that antibodies dir cted against the cell binding site of tenascin, SRRGDMS, mimic the tenascin receptor and compete with the cellular receptor for tenascin binding sites. These anti-peptide antibodies recognize both intact tenascin as well as the peptide to which it was raised but not GRGDSP-BSA. The specificity of the interaction with SRRGDMS-BSA and not GRGDSP-BSA confirms that the epitopes are unique. These results demonstrate that the anti-peptide antibody is binding to the cell attachment site of tenascin which is distinct from that in fibronectin.

The specificity of antibody inhibition contrasts with the results of peptide inhibition in Examples 4 and 5 in which either SRRGDMS or GRGDSP peptide were effective in inhibiting tenascin cell attachment. The likely explanation for the less selective properties of peptides is that the antibodies recognize a particular structural conformation, while the peptides may assume large number of conformations capable of interacting with integrin receptors. The results of anti peptide antibody cell attachment inhibition also imply that these antibody may be used to selectively block tenascin activities mediated by the SRRGDMS site.

What is claimed is:

1. A polypeptide of the formula:

X-Ser-Arg-Arg-Gly-Asp-Met-B-Z

wherein:

B is Ser or Glu;

X is a chain of 1 to 20 amino acid residues or an amino-terminal group; and

Z is a chain of 1 to 20 amino acid residues or a carboxy-terminal group.

2. The polypeptide of claim 1 wherein X is selected from the group consisting of:

(a) Gly-Arg-Gly-Asp-Ser-Pro,

(b) Arg-Gly-Asp-Thr, and

(c) Arg-Gly-Asp-Cys.

3. The polypeptide of claim 1 wherein Z is selected from the group consisting of:

(a) Gly-Arg-Gly-Asp-Ser-Pro,

(b) Arg-Gly-Asp-Thr, and

(c) Arg-Gly-Asp-Cys.

4. The polypeptide of claim 1 wherein said carboxy-terminal group is NH_2.

5. The polypeptide of claim 1 attached to a solid matrix.

6. The polypeptide of claim 5 wherein said solid matrix is comprised of collagen.

7. The polypeptide of claim 5 wherein said solid matrix is comprised of nitrocellulose or polyester.

8. The polypeptide of claim 5 wherein said solid matrix is glass, synthetic resin or long chain polysaccharide.

9. The polypeptide of claim 5 wherein said solid matrix is a synthetic resin fiber.

10. The polypeptide of claim 1 attached to the surface of a prosthetic device.

11. The polypeptide f claim 1 attached to the surface of a percutaneous d vice.

12. The polypeptide of claim 1 coupled to and forming part of a vascular graft.

13. The polypeptide of claim 1 in the form of a lotion, salve, gel, colloid, or powder.

14. A polypeptide having the formula:

NH_2 -Ser-Arg-Arg-Gly-Asp-Met-Ser-$_p$ COOH

wherein p is an integer having a value such that the polypeptide is soluble in aqueous 0.15 M sodium chloride.

15. The polypeptide of claim 14 wherein p has a value of 2 to 6.

16. A polypeptide of the formula:

NH_2-Ser-Arg-Arg-Gly-Asp-Met-Ser-COOH,

characterized in that it has cell attachment promoting activity.

17. A polypeptide of the formula:

NH_2 Ser-Arg-Arg-Gly-Asp-Met-Ser-NH_2,

characterized in that it has cell attachment promoting activity.

18. A polypeptide having less than about 30 amino acid residues and a biologically active sequence exhibiting cell attachment activity, said sequence represented by the formula:

Ser-Arg-Arg-Gly-Asp-Met-Ser

19. A method for inhibiting attachment of cells to tenascin comprising contacting the cells with a peptide according to claim 1 in an amount of said peptide effective to inhibit said attachment.

20. A method for inhibiting attachment of cells to a subcellular matrix comprising contacting the subcellular matrix with an antibody that immunoreacts with a polypeptide of the formula:

Ser-Arg-Arg-Gly-Asp-Met-Ser,

but does not immunoreact with a polypeptide f the formula:

Gly-Arg-Asp-Gly-Ser-Pro.

21. The method of claim 20 wherein said antibody is a monoclonal antibody.

22. A method for enhancing cell attachment to a solid matrix comprising operatively linking a polypeptide according to claim 1 to said solid matrix.

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                                                    G  1101

1102  GTGACCACCACACGCTTGGATGCCCCCAGCCAGATCGAGGTGAAAGATGTC
      V  T  T  T  R  L  D  A  P  S  Q  I  E  V  K  D  V

1153  ACAGACACCACTGCCTTGATCACCTGGTTCAAGCCCCTGGCTGAGATC
      T  D  T  T  A  L  I  T  W  F  K  P  L  A  E  I

1201  GATGGCATTGAGCTGACCTACGGCATCAAAGACGTGCCAGGAGACCGTACC
      D  G  I  E  L  T  Y  G  I  K  D  V  P  G  D  R  T

1252  ACCATCGATCTCACAGAGGACGAGAACCAGTACTCCATCGGGAACCTG
      T  I  D  L  T  E  D  N  Q  Y  S  I  G  N  L  K

1300  AAGCCTGACACTGAGTACGAGGTGTCCCTCATCTCCCGCAGAGGTGACATG
      K  P  D  T  E  Y  E  V  S  L  I  S  R  R  G  D  M

1351  TCAAGCAACCCAGCCAAAGAGACCTTCACAACAGGCCTCGATGCTCCC
      S  S  N  P  A  K  E  T  F  T  T  G  L  D  A  P

1399  AGGAATCTTCGACGTGTTTCCCAGACAGATAACAGCATCACCCTGGAATGG
      R  N  L  R  R  V  S  Q  T  D  N  S  I  T  L  E  W

1450  AGGAATGGCAAGGCAGCTATTGACAGTTACAGAATTAAGTATGCCCCC
      R  N  G  K  A  A  I  D  S  Y  R  I  K  Y  A  P

1498  ATCTCTGGAGGGGACCACGCTGAGGTTGATGTTCCAAAGAGCCAACAAGCC
      I  S  G  G  D  H  A  E  V  D  V  P  K  S  Q  Q  A

1549  ACAACCAAAACCACACTCACAGGTCTGAGGCCGGGAACTGAATATGGGATT
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1600  G
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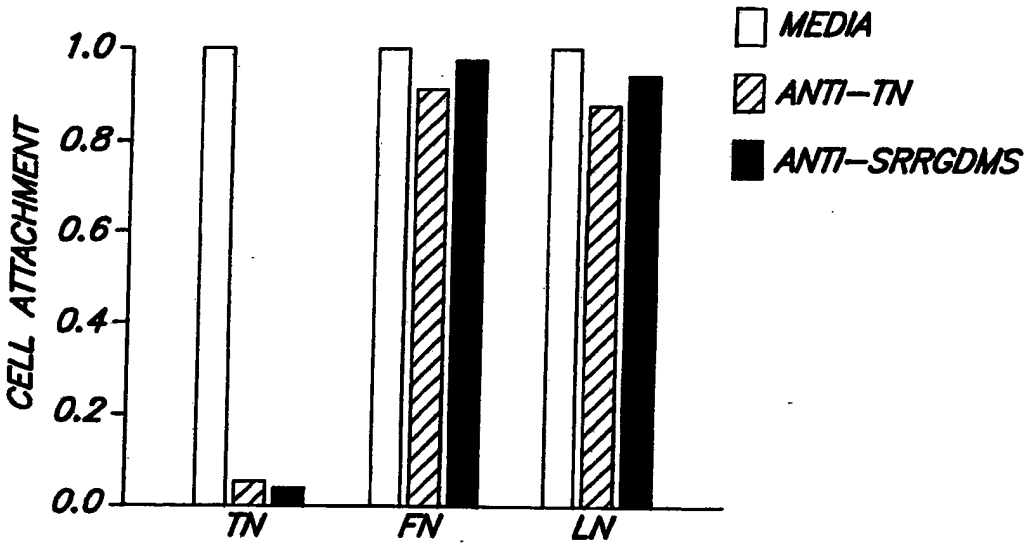
FIG. 1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ANTI–TN
ANTI–SRRGDMS
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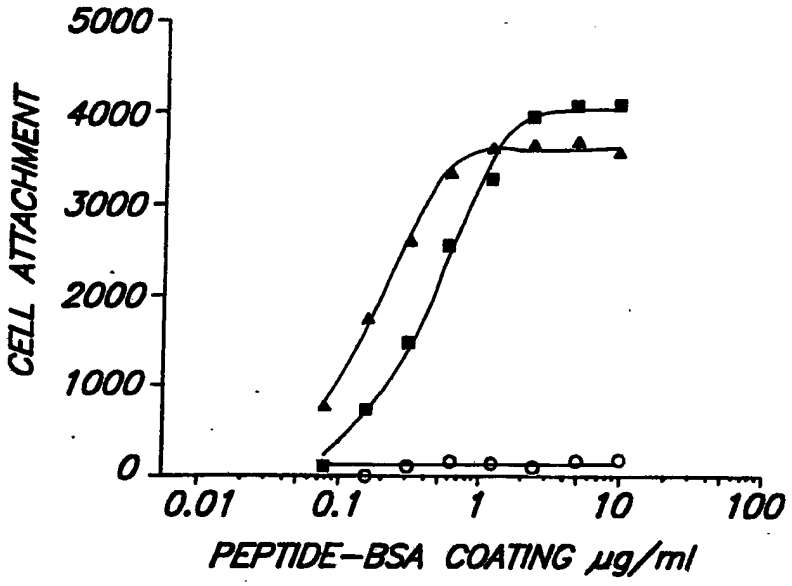
TN
FN
LN

FIG. 2


CELL ATTACHMENT
5000
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2000
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0
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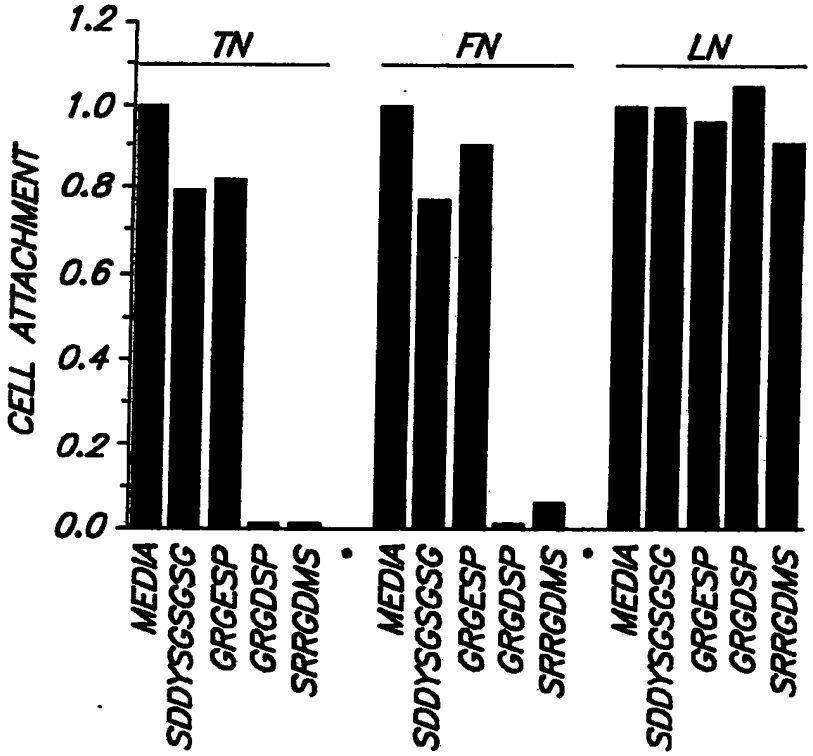
1
10
100
PEPTIDE–BSA COATING µg/ml

FIG. 3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FN
LN
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GRGESP
GRGDSP
SRRGDMS
MEDIA
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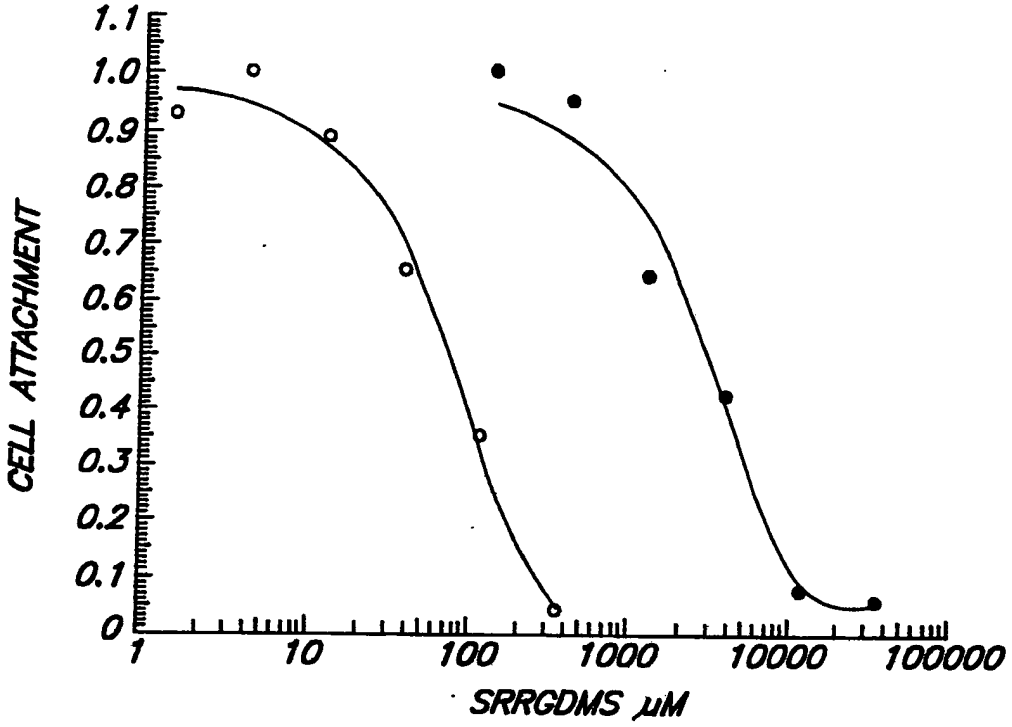
GRGDSP
SRRGDMS

FIG. 4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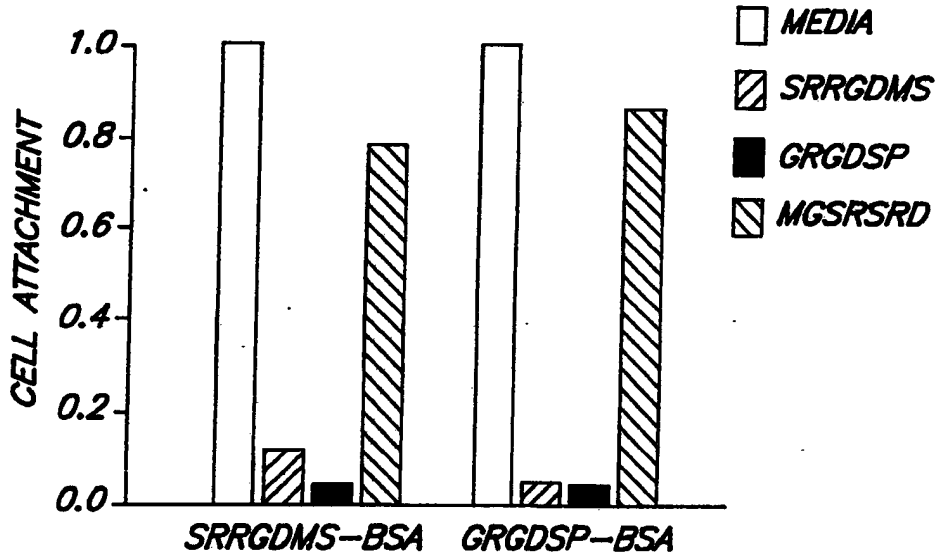
1
10
100
1000
10000
100000
SRRGDMS μM

FIG. 5


CELL ATTACHMENT
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0.8
0.6
0.4
0.2
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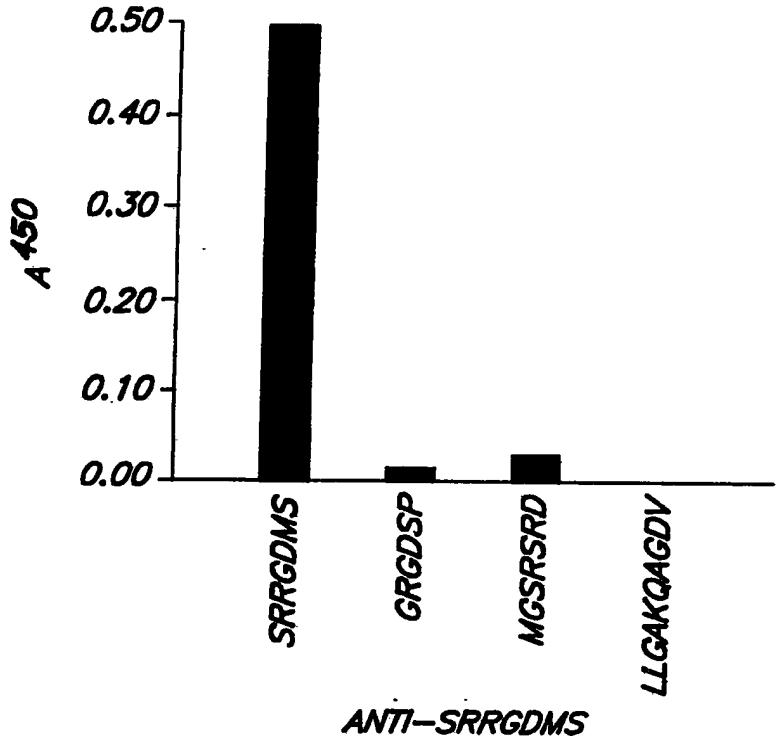
SRRGDMS–BSA
GRGDSP–BSA
MEDIA
SRRGDMS
GRGDSP
MGSRSRD

FIG. 6

FIG. 7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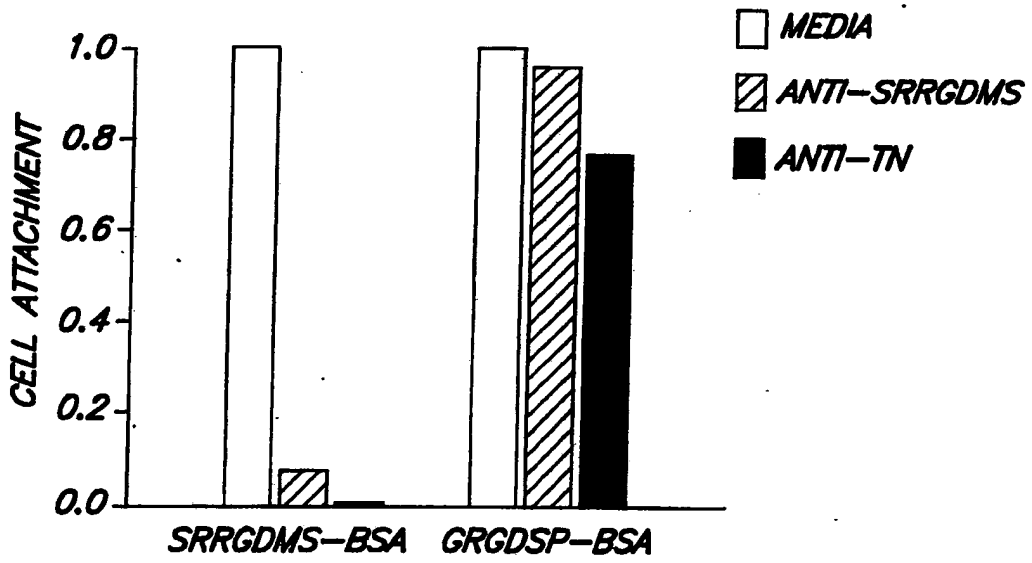
GRGDSP
MGSRSRD
LLGAKQAGDV
ANTI-SRRGDMS


1.0
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0.6
0.4
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0.0
CELL ATTACHMENT
SRRGDMS-BSA
GRGDSP-BSA
MEDIA
ANTI-SRRGDMS
ANTI-TN

FIG. 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08018

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07K 7/06,7/08,7/10;A61K 37/02,39/00
US CL : 530/328,327,326,325,324;514/15,14,13,12;424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	530/328, 327, 326, 325, 324; 514/15, 14, 13, 12; 424/85.8

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

SEQUENCE SEARCH IN CHEMICAL ABSTRACTS REGISTRY FILE AND SWISS-PROT, PIR, AND GENESEQ DATABASES. TEXT SEARCH IN CAS, MEDLINE, AND BIOSIS DATABASES.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,638,291 (ZIMMERMAN ET AL.) 28 July 1987. See especially the abstract and col. 1.	1-5,16-18
A	CELL, Volume 44, issued 28 February 1986, E. Ruoslahti et al., "Arg-Gly-Asp: A Versatile Cell Recognition Signal", pages 517-518. See especially col. 1.	1-5,16-18
A	US, A, 4,614,517 (RUOSLAHTI ET AL.) 30 September 1986.	1-22
A	US, A, 4,857,508 (ADAMS ET AL.) 15 August 1989.	1-22
A	Science, Volume 238, issued 23 October 1987, E. Ruoslahti et al., "New Perspectives in Cell Adhesion: RGD and Integrins", pages 491-497.	1-5,16-18

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
11 FEBRUARY 1992	25 FEB 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	SUSAN M. PERKINS